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Environmental concentrations of prednisolone alter visually mediated responses during early life stages of zebrafish (*Danio rerio*)

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Abstract

The development of the eye in vertebrates is dependent upon glucocorticoid signalling, however, specific components of the eye are sensitive to synthetic glucocorticoids. The presence of synthetic glucocorticoids within the aquatic environment may therefore have important consequences for fish, which are heavily reliant upon vision for mediating several key behaviours. The potential ethological impact of synthetic glucocorticoid oculotoxicity however has yet to be studied. Physiological and behavioural responses which are dependent upon vision were selected to investigate the possible toxicity of prednisolone, a commonly occurring synthetic glucocorticoid within the environment, during early life stages of zebrafish. Although exposure to prednisolone did not alter the morphology of the external eye, aggregation of melanin within the skin in response to increasing light levels was impeded and embryos exposed to prednisolone (10 µg/l) maintained a darkened phenotype. Exposure to prednisolone also increased the preference of embryos for a dark environment within a light dark box test in a concentration dependent manner. However the ability of embryos to detect motion appeared unaffected by prednisolone. Therefore, while significant effects were detected in several processes mediated by vision, changes occurred in a manner which suggest that vision was in itself unaffected by prednisolone. Neurological and endocrinological changes during early ontogeny are considered as likely candidates for future investigation.

Capsule

Exposure to prednisolone alters physiological and behavioural responses to visual stimuli during zebrafish embryogenesis via proposed non-ocular mechanisms.

Keywords

Prednisolone, zebrafish, embryo, vision, behaviour

Introduction

Autoimmune and inflammatory conditions e.g. asthma and arthritis, are routinely treated using a variety of synthetic glucocorticoid based therapies. However, these treatments are commonly associated with an increased rate of visual defects, including the development of cataracts and glaucoma.[1] Exposure to synthetic glucocorticoids also affects specific ocular tissues *in vitro* and subsequently induces a range of abnormalities. Increased opacity of the lens, proptosis, unfused eyelids, epithelial defects at the lens and cornea, ocular hypotension and changes to the structure of trabecular meshwork cells are associated with synthetic glucocorticoid toxicity.[2,3] The introduction of synthetic glucocorticoids within the aquatic environment[4,5,6] via waste water effluent may therefore have a significant impact upon the visual system of fish which are dependent upon glucocorticoid signalling to mediate ocular development. Knockout of the GR transcript in zebrafish (*Danio rerio*) embryos causes a significant increase in crystalline proteins associated with development of the lens.[7] GR morphants also display temporal changes in *sox9b* expression which plays a significant role in regulating the number of Müller glia and photoreceptor cells during neural retinogenesis in zebrafish.[8] MMP14a, a matrix metalloproteinase (MMP), helps regulate retinal and retinotectal development[9], however, MMP expression is significantly affected by synthetic glucocorticoids in zebrafish[10-12]. Recent evidence has shown that prednisolone, a commonly detected synthetic glucocorticoid in the environment, significantly alters physiological processes during zebrafish development in a manner consistent with the effects of endogenous glucocorticoid signalling[13]. The potential behavioural impact of synthetic glucocorticoids on the visual system during ontogeny however has yet to be investigated in fish.

Zebrafish provide an excellent model system to explore the potential ethological effects of synthetic glucocorticoids on the visual system. The development of the eye in zebrafish has already received considerable attention as a model to investigate visual processes and genetic disease in vertebrates.[14-16] As such, the development of the eye[17-21], the ontogeny of vision[22-27] and the onset of associated behaviours[27] have been well categorised in zebrafish embryos and larvae.

Behaviour has been increasingly utilised as a sensitive indicator of developmental change in response to contaminants[29] and changes to visually mediated behaviours have been used extensively to assess the oculotoxicity of several contaminants in early life stages of zebrafish[30-33]. The eye first appears at around 10 hpf in zebrafish and the first visually mediated behaviours are evident from 70 hpf[19] which facilitates rapid toxicological screening as several physiological and behavioural responses are principally mediated by vision during embryogenesis.

The ability to alter skin pigmentation in response to changing light levels is believed to facilitate anti-predatory behaviour.[16] This process is regulated by photoreceptors in the eye as well as those within the pineal gland and melanophores, however, the aggregation of pigment is principally mediated by vision[34]. An absence of photoreceptors in the retina of *eby* and *ivy* zebrafish mutants is associated with an inability to aggregate melanin within the melanophores, resulting in extreme hyper-pigmented phenotypes.[35] Similarly, the *lakritz* zebrafish mutant contains 80% less retinal ganglion cells within the retina, which are responsible for transmitting visual information to various areas of the brain, resulting in a darkened phenotype.[15] Changes to the composition of the retina, in response to synthetic glucocorticoids, may therefore manifest externally during the light adaptation response.

Changes to light sensitivity may also impact behaviour. The light-dark box test (LDBT), which has predominantly served as a means to assess anxiety, has been successfully applied in rodent models to evaluate differences in visually mediated behaviours[36]. Based upon an innate preference for either light or dark areas depending upon the design of the apparatus[37], changes in preference for light over dark areas is hypothesised to facilitate vision[38]. Changes in the eye's sensitivity to light may also be expected to reduce the perceived difference between light and dark areas. Similar tests have been used to investigate ocular toxicity in zebrafish.[31]

The ability to detect motion develops early during zebrafish ontogeny and is necessary to allow prey tracking behaviour and predatory detection.[39,40] Zebrafish embryos display characteristic avoidance behaviour in response to a moving object and may therefore be utilised to evaluate the ability of zebrafish embryos to detect motion. Pelkowski *et al*.[39] and Richendrfer and Créton[41] used simple animations consisting of basic shapes to induce avoidance reactions in early life stages of zebrafish. The ability to perceive movement

requires the capacity to form images and to track their change in position over time and requires advanced visual processes which function in conjunction with the neurological system. Abnormal behavioural responses to moving stimuli have previously highlighted specific mutations in the neural circuitry and composition of the retina in zebrafish larvae.[15]

Investigating the developmental effects of synthetic glucocorticoids on the visual system may not only reveal the impact of this class of compounds within an environmental context, but may also reveal specific sensory and neurological targets of synthetic glucocorticoids during early ontogeny in vertebrates. This study therefore investigated the effects of prednisolone on morphological, physiological and behavioural responses specifically associated with vision during early development in zebrafish. All experimental animals are herein referred to as embryos, in line with Balon's classification of early life stages, as all were pre-first feeding[42].

Methods

Embryo collection and prednisolone exposure

Adult zebrafish from an existing stock at the University of the West of Scotland (UWS) were maintained in 25 l glass aquaria (28±1°C; 12:12 light-dark) in a 400 l re-circulatory system. Fish were fed twice daily with flake (AQUARIAN) and once daily with *Artemia* sp. nauplii *ad libitum*. Males and females were separated prior to spawning when they were mixed within a breeding net, allowing collection of embryos. Embryos were maintained in 50 ml of embryo medium (5 mM NaCl, 0.17 mM KCl, 0.33 mM $MgSO_4.7H_20$, 0.33 mM $CaCl_2.2H_2O$ and 10^{-5}% of methylene blue in distilled water)[43] in 100 ml beakers kept in water baths (28±1°C; 8:16 light-dark). Any embryos which failed to develop normally during the experiment were removed daily. Groups of 50 embryos were exposed to 0.1, 1 and 10 µg/l of prednisolone (Sigma) dissolved in ethanol (0.01%, Analytical grade, Sigma) and were continually exposed following fertilisation (< 1 h) using static exposures with partial renewal (50%) every 24 h. Stock solutions were made daily prior to use and were kept at 4°C in the dark. Water and solvent controls were also tested. Experimental treatments were replicated four times (i.e. four beakers of each treatment) for morphometric analysis and three times for all other measurements.

Chemical analysis

Water samples were analysed at the University of Santiago de Compostela (USC), Spain *via* liquid chromatography-tandem mass spectrometry (LC-MS/MS) using the previously published method for analysing pharmaceuticals including corticosteroids[44]. Please refer to McNeil *et al*. 2016[13] for sample preparation and experimental conditions used. Concentrations of samples collected at 0 h were 0.1±0.05, 1.1±0.63 and 8.2±0.45 µg/l. No prednisolone was detected within either control group. Due to sample degradation while in transport between UWS and USC, chemical analysis of water samples collected at later time points is not provided as all samples were affected. Results are therefore discussed in terms of nominal concentrations of prednisolone at 0 h.

Morphometric analysis

Morphometric analysis of the outer eye was conducted at 72, 96 & 120 hpf. Embryos were anaesthetised in MS-222 and were photographed using a Leica MZFIII photo-microscope, connected to a digital camera. Images were then viewed in Image J.[45] The area of the entire eye and pupil were calculated. Four embryos from four treatment replicates were photographed (n = 16). Preliminary tests were also conducted to investigate the potential changes to the opacity of the lens following prednisolone treatment. Lenses were removed from embryos at 120 hpf and were viewed microscopically. No visible morphological differences or changes to opacity were observed in response to any concentration of prednisolone and so no further analysis was conducted.

Light adaptation

Methods were modified from Shiraki *et al*.[34] Embryos (96 hpf) were acclimated to darkness for 3 h during the 3rd hour of their light cycle. This ensured all embryos had fully acclimated to darkness and reduced variation in skin pigmentation due to circadian rhythmicity. Embryos were mounted in 6% methylcellulose on microscope slides and were then returned to darkness for 30 min. Embryos were then positioned above a light source (500 lux, measured using a Dr. Meter LX1010BS digital lux meter) underneath a Leica MZFIII photo-microscope connected to a digital camera. From here, a specific area of the embryo (Figure 1) was photographed at 0, 20 and 40 minutes post-light exposure. When not being photographed, embryos were continuously exposed to light conditions (500 lux). The change in the area of pigmentation was measured over time using Image J and compared between treatments. Four embryos from three treatment replicates were photographed (n = 12).

Light-dark box test

The light-dark box test was conducted at 120 hpf. The apparatus consisted of a 6 cm diameter petri dish marked with a grid floor (4.5 mm^2; approx. one body length), equally divided into a white and black area. The floor of the dish was further divided into three circular areas (outer, middle & central). Individual embryos were initially acclimated within a small transparent chamber positioned within the central area for 1 minute before being allowed to explore the tank for 5 minutes. Behaviour was filmed using a JVC TH-C1480B colour camera positioned directly above the test apparatus. The entire apparatus and camera were screened from view using a curtain to prevent disturbance. The percentage of time spent in each compartment (light vs. dark), the proportion of time within each of the three circular areas within the light compartment and the total level of activity (total number of lines crossed) while in the white compartment was recorded. Four larvae from three treatment replicates were observed (n = 12).

Avoidance of visual stimulus

Avoidance behaviour was tested at 144 hpf in response to an animated visual stimulus. Embryos were placed into individual wells of a six well plate containing embryo medium (28°C). The plate was positioned on top of a horizontal laptop screen and was filmed from above using a JVC TH-C1480B colour camera connected to a desktop PC. The visual stimulus was produced *via* the laptop using Microsoft PowerPoint and consisted of two black rectangles (0.4 x 14.6 cm) which were positioned parallel to one another. Each rectangle repeatedly moved forwards and backwards between the inner edge of one row of wells and the centre of the well (Figure 2), separating each well into an area with and without a moving black bar. Individuals were allowed to acclimate for 5 minutes on top of a white screen and then were recorded for 5 minutes before the presentation of the stimulus began. The animation was then played for 5 minutes. Afterwards, embryos were once again presented with a blank white background for 5 minutes. The amount of time embryos spent in the outer area of the well, which contained no visual stimulus, was calculated for each of three 5 minute observation periods (pre-animation, during and post-animation). The latency to leave the inner area after the onset of the animation and the latency to re-enter the inner area after completion of the animation was also recorded. Methods were based on Richendrfer and Créton[41] and were validated using a pilot study which found that the visual stimulus produced

a significant avoidance reaction. Six embryos from three treatment replicates were observed (n = 18).

Statistical analysis

All statistical analysis was conducted using SPSS v.18. Data were checked for normality and homogeneity of variances using Kolmogorov-Smirnov and Levene's tests, respectively. Where data were not normally distributed, or in the case of percentage data, transformations were conducted to allow parametric testing by ANOVA with Fisher's LSD test post hoc. Non-parametric equivalents were used where necessary (Scheirer-Ray-Hare (SRH), Kruskal-Wallis test (KW) and pairwise Mann-Whitney U). No effect of beaker replicate was found for any of the variables tested and so replicates were combined for statistical analysis.

Results

Prednisolone exposure did not induce any superficial malformations during ontogeny at any of the observed stages of development. Nor did prednisolone treatment significantly affect the area of the entire eye or the area of the pupil.

Exposure to all three concentrations of prednisolone resulted in significantly darker embryos from the onset of the light adaptation procedure (2-way ANOVA: Time, $P < 0.001$, Treatment, $P < 0.001$: Time*Treatment, $P = 0.262$, Figure 3).However, only those embryos exposed to 10 µg/l of prednisolone produced a sustained darkened phenotype throughout the entire 40 minutes of light exposure. There was no significant difference in the overall change in the area of pigmentation over the 40 minutes between treatments.

Prednisolone also significantly affected the proportion of time spent in the dark area of the LDBT (ANOVA, $P < 0.001$, Figure 4). While exposure to 0.1 µg/l of prednisolone significantly reduced the percentage of time spent in the dark area, 10.0 µg/l of prednisolone significantly increased the proportion of time spent in the dark area. Exposure to 1.0 µg/l did not produce any significant change in preference compared to controls. Activity and the percentage of time spent in each of the three areas within the light compartment did not significantly vary between treatments.

Individuals significantly increased the amount of time spent away from the moving image in the outer area of the plate (ANOVA, $P < 0.001$, Figure 5). No significant difference however was found between treatments. There were also no significant differences in the latency to move away from the moving stimulus (KW, $\chi^2_{(4)} = 2.521$, $P = 0.641$) or in the latency to re-enter the area in which presented the stimulus (KW, $\chi^2_{(4)} = 1.552$, $P = 0.817$).

Discussion

Exposure to environmentally relevant concentrations of prednisolone resulted in significant changes to physiological and behavioural parameters associated with vision during embryogenesis in zebrafish. However, evidence suggests these changes are not mediated by changes to vision directly. No superficial malformations or morphological changes were observed in the eyes of embryos exposed to any concentration of prednisolone.

However, embryos exposed to 10 µg/l of prednisolone retained a significantly darker phenotype throughout the light adaptation period (Figure 3). Since the overall reduction in the area of dispersed pigment was not significantly different between treatments during the 40 minute light exposure, embryos appear to be able to mediate skin colour at a rate comparable to controls, which suggests that the detection of light *via* photoreceptors within the eye is not detrimentally affected by prednisolone. Therefore the darkened phenotype of embryos exposed to 10 µg/l of prednisolone does not appear to be related to effects associated with vision but may in fact be related to changes to the melanogenic system. Melanin-concentrating hormone (MCH) is responsible for the aggregation of melanin and works in conjunction with alpha-melanocyte stimulating hormone (αMSH) to regulate skin colour.[46] As well as mediating camouflage, skin colour also facilitates visual cues between conspecifics which allows individuals to mediate agonistic relationships while reducing costs to fitness. Under these circumstances, pigmentation is controlled by the hypothalamic-pituitary-interrenal (HPI) axis which controls the physiological response to stress[47]. Endogenous glucocorticoids are therefore involved in controlling the aggregation and dispersal of melanin. Zebrafish mutants which lack functional GR show an impaired background adaptation response i.e. remain darker on a white background which coincides

with an up-regulation of αMSH.[48] Betamethasone17-valerate, a synthetic glucocorticoid, suppresses the pro-opiomelanocortin (POMC) transcript in wild type zebrafish, which is the precursor of αMSH. Corticotropin-releasing factor (CRF) is also involved in the control of melanin within the melanophores of zebrafish embryos.[49] Therefore, exposure to synthetic glucocorticoids may alter skin pigmentation *via* changes to those hormones associated with regulating melanin transport and so exposure to 10 µg/l of prednisolone may mediate changes to skin colouration by altering HPI activity.

While the aggregation of melanin is mediated by vision, the dispersal of melanin is regulated by photoreceptors found directly within the melanophores[34]. Exposure to 0.1 and 1 µg/l of prednisolone increased the area of dispersed melanin during the dark adaptation period but both treatments resembled controls after the first 20 minutes of the light adaptation period. This increase in dispersed melanin may be associated with changes to the photoreceptors within the melanophores. Alternatively, it could also be linked to the hypothesised changes to the HPI axis.

Results from the LDBT show that control embryos prefer light over dark environments (Figure 4). Zebrafish embryos lose vision at night due to circadian control over retinal sensitivity[50] and not due to the onset of darkness. Therefore the retina should remain active during the 3 hour dark adaptation period. However, zebrafish embryos do not yet possess functional rods within the retina[51], which are responsible for vision during low light intensities[52], and so while the retina may remain active, the embryos are functionally blind during this period. This could represent a significant stressor to embryos and a subsequent increase in circulating cortisol which may coincide with changes to melanogenic hormones. Differences between these lower concentrations of prednisolone and control embryos may also suggest that HPI functioning has been altered. Changes in the regulation of those hormones associated with the HPI axis may also be expected to significantly alter behaviour and may be linked to those observed changes in the LDBT.

Overall, control embryos displayed a clear preference for the light compartment in the LDBT which is consistent with previous studies.[38,53-55] However, the percentage of time embryos spent in the dark compartment was significantly altered in response to prednisolone (Figure 4). Exposure to 0.1 µg/l of prednisolone significantly increased dark avoidance compared to controls, while 10 µg/l prednisolone of resulted in a significant increase in the preference for

darkness. Given that the size of the pupils was not significantly different, nor were there any differences in lens opacity, the amount and quality of light entering the eye is not hypothesised to differ between treatments. However, changes to the organisation of the retina and altered photoreceptor development may alter the detection and conversion of light into chemical and electrical signals. Thompson *et al.*[36] showed that mice which display reduced non-image-forming irradiance detection (*Rpe65−/−*) and which exhibit loss of rod and cone functionality (*rd1*), increase scotophobia during the LDBT. Yet, since embryos exposed to prednisolone displayed a light adaptation response in a manner consistent with controls, the processing of light cues appears to remain intact.

Changes in the preference for either light or dark areas are more commonly associated with changes to neurological function and have become a popularised method to define the neurophenotype of individuals.[56] Variation in how different individuals respond to such stimuli has led to the development of behavioural syndromes, which are defined as a collection of behavioural responses which are consistent over time and between different contexts.[57] Several aspects of behaviour are used as indicators of behavioural syndromes, including anxiety[58], which may occur in response to a perceived threat or in response to novelty[59]. However understanding the underlying motivations behind preference is complex and contrasting results between studies further confounds the interpretation of behaviour during the LDBT. While some studies have linked increased preference for darkness with increased levels of anxiety[60], others have found a preference for brighter areas aids in alleviating stress[38]. Experimental conditions are therefore an important factor in interpreting preference (reviewed by Maximino *et al.*[37]). In the present study, embryos were only acclimated for a 1 minute period and so individuals may have remained stressed during the LDBT since they were in a novel environment. Therefore, preference should reflect a strategy to help reduce anxiety.

Control embryos spent 90% of their time within the light compartment which would suggest that avoiding dark areas helps to alleviate stress in this particular novel environment. Exposure to 0.1 µg/l of prednisolone significantly increased the proportion of time spent in the light compartment compared to controls, which would therefore suggest increased anxiety. In contrast, exposure to 1 and 10 µg/l of prednisolone reduced the proportion of time in the light compartment and may be interpreted as reduction in anxiety. Therefore, results suggest that prednisolone alters the preference for light and dark areas and may be associated

with changes to neurological development. This could further implicate the HPI axis as a probable target of prednisolone toxicity. Anxiogenic compounds are known to induce behavioural responses synonymous with stress without simultaneously affecting multiple indices of anxiety.[60] This may explain why activity and thigmotaxis did not vary between treatments during the LDBT in this study. Preference for light and dark areas may provide a more consistent measure of anxiety in response to synthetic glucocorticoid exposure.

Exposure to the animated stimulus resulted in a significant change in position away from the moving image (Figure 5) which is consistent with Pelkowski *et al*.[39] and Richendrfer and Créton[41] who reported avoidance behaviour in response to visual stimuli during early life stages of zebrafish. No significant difference was found in the amount of time embryos spent away from the moving image between any of the concentrations of prednisolone tested however. Therefore, the ability to detect motion appears to be unaffected by prednisolone at these concentrations. Combined with evidence from the light adaption response and the LDBT, it is hypothesised that prednisolone does not alter vision in zebrafish embryos at environmentally relevant concentrations. Interestingly, results from the visual avoidance test would also suggest that embryos exposed to prednisolone respond to fear inducing stimuli in a manner similar to controls unlike during the LDBT, which triggered differences in behaviours associated with anxiety. Fear and anxiety are two distinct behavioural responses; while anxiety relates to perceived threat, fear is a response to an immediate risk. Both types of behavioural response utilise specific regions of the brain; the medial region of the dorsal pallium is believed to be the teleost equivalent of the mammalian amygdala which mediates fear responses, while the media habenula is associated with regulating behaviours indicative of anxiety.[59] Therefore differences in anxiety but not fear may suggest specific neurological effects.

Further studies should therefore attempt to investigate the neurological impact of prednisolone at environmentally relevant concentrations. Dexamethasone, another potent synthetic GR agonist, is known to down regulate GR expression in rainbow trout (*Oncorhynchus mykiss*) brains.[61] Differences in mineralocorticoid receptor expression, which also bind glucocorticoids in teleosts, have also been shown to exist between individuals which display alternative behavioural phenotypes.[62] Understanding the underlying molecular and physiological HPI response following prednisolone exposure may reveal possible underlying toxic mechanisms responsible for the observed changes to behaviour.

In conclusion, exposure to environmentally relevant concentrations of prednisolone does not appear to affect the detection of light or the ability to detect motion. However, prednisolone exposure produced a darkened phenotype which coincided with a change in preference for darker environments. This change in pigmentation persisted within a light environment and suggests that this change may be maladaptive and may be associated with changes to the melanogenic system. If scototaxis is a reliable indicator of anxiety in embryos, this would suggest that prednisolone mimics the action of cortisol in adult zebrafish which predisposes individuals to different behavioural syndromes i.e. darker males are more aggressive[63]. Whether a reduction in anxiety and changes in skin pigmentation are also associated with changes in aggression and additional behavioural measures of copying style requires further attention. However, increased preference of dark environments is associated with boldness which tends to coincide with aggressiveness in fish.[58] Differences between anxiety and fear responses also advocate neurological changes in specific regions of the developing brain. How and when such changes occur and how they may alter performance and survival now requires investigation.

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Figure legends

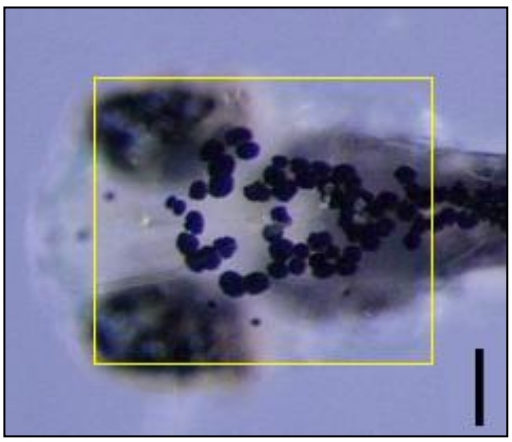
Figure 1: Photograph of an embryo at 96 hpf. Yellow line outlines the area analysed during light adaptation. Scale bar = 200 µm.

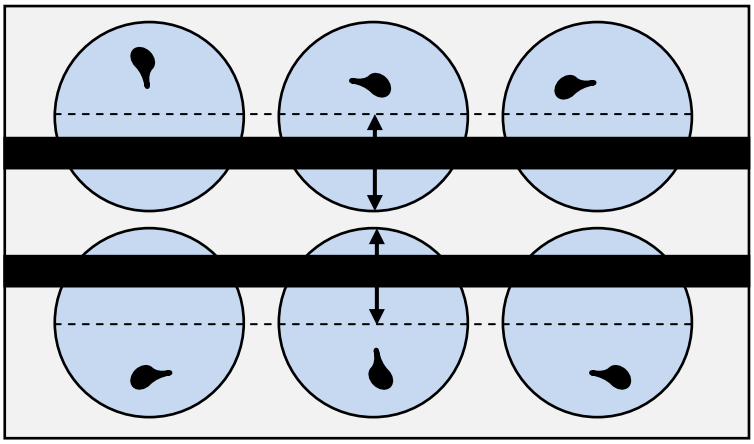
Figure 2: Diagram of the avoidance response assay. Arrows denote the direction of each of the moving black bars and the dashed line marks the range of the animation.

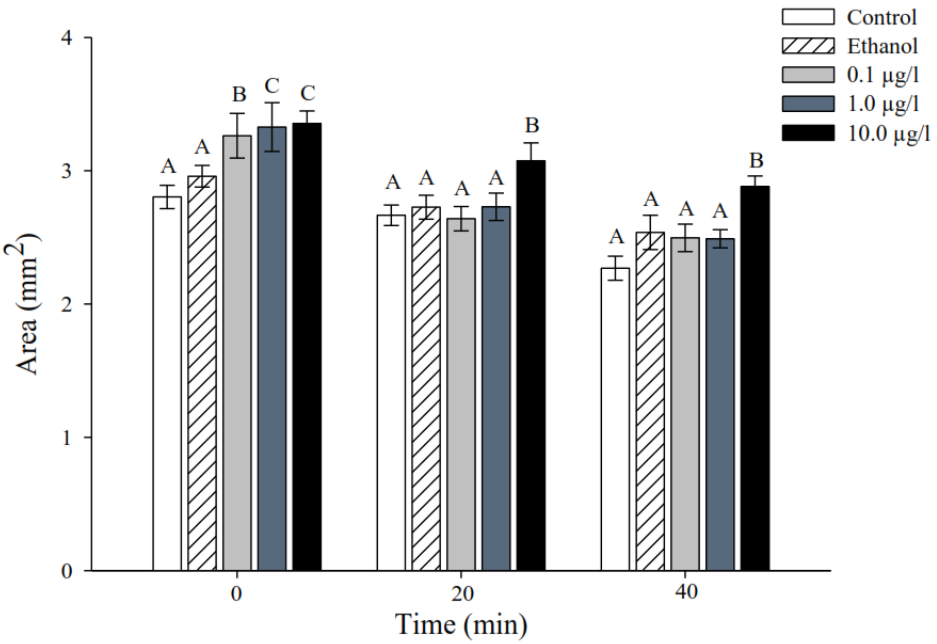
Figure 3: Area of pigmentation during light adaptation at 96 hpf (n = 12). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Means ± SEM.

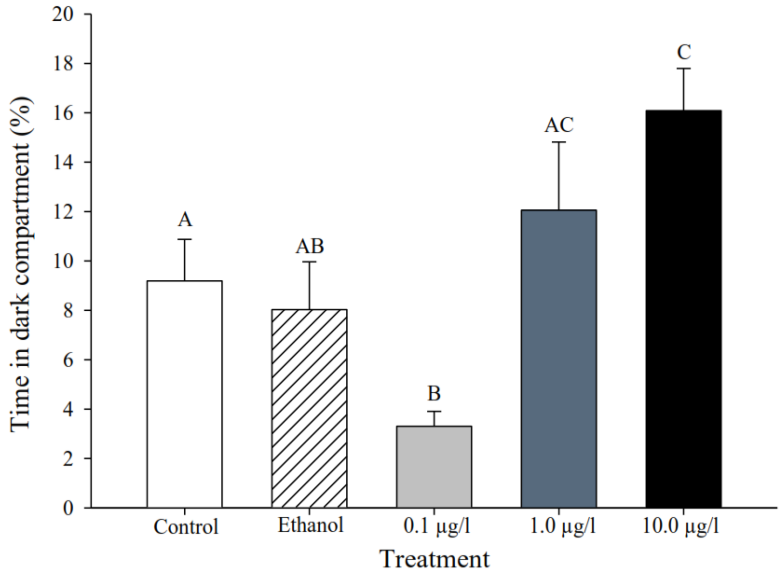
Figure 4: Percentage of time spent in the dark area of the LDBT at 120 hpf (n = 12). Letters denote significant differences between treatments at specific time points ($P < 0.05$). Where bars share letters there is no significant difference. Means ± SEM.

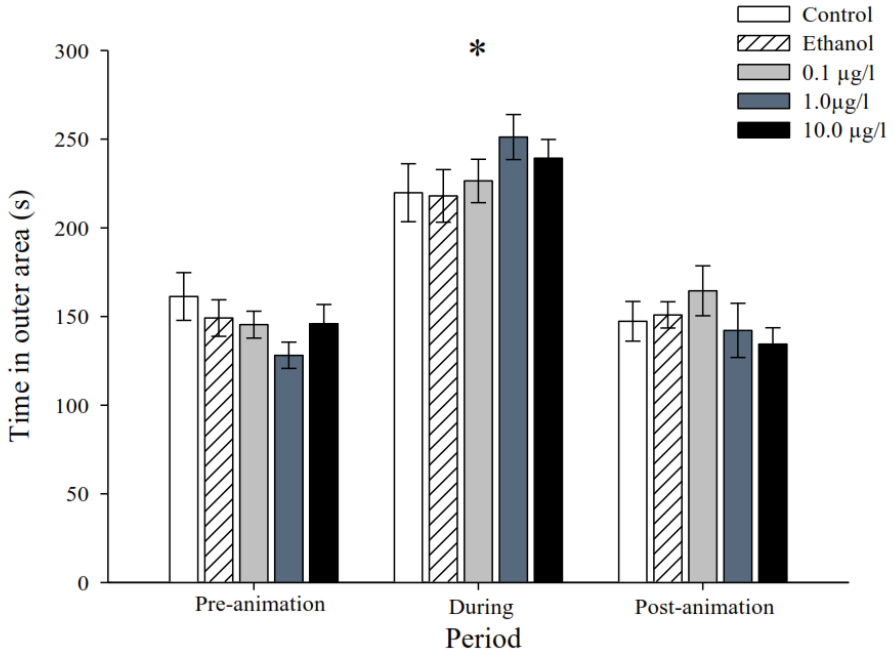
Figure 5: Time spent in the outer area of the well plate during the avoidance response assay in seconds at 144 hpf (n = 18). Asterisks denote significant differences between different time periods. Means ± SEM.






Control
Ethanol
0.1 µg/l
1.0 µg/l
10.0 µg/l
Area (mm2)
Time (min)
0
20
40
A
A
B
C
C
A
A
A
A
B
A
A
A
A
B


Time in dark compartment (%)
20
18
16
14
12
10
8
6
4
2
0
A
AB
B
AC
C
Control
Ethanol
0.1 µg/l
1.0 µg/l
10.0 µg/l
Treatment


Control
Ethanol
0.1 µg/l
1.0µg/l
10.0 µg/l
*
300
250
200
150
100
50
0
Time in outer area (s)
Pre-animation
During
Post-animation
Period

734